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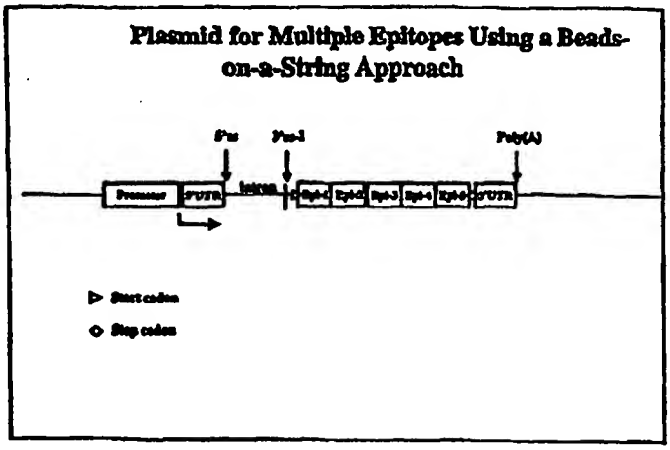

(57) Abstract

Improved plasmids and methods for nucleic acid–based vaccines. The use of epitopes, small immunologically relevant protein sequences that are capable of inducing both cellular and humoral responses that result in a protective or therapeutic immune response against large and complex pathogens for incorporation into nucleic acid–based vaccines. The structures and characteristics of gene expression systems that are capable of expressing multiple epitopes. A method of genetic immunization comprising the step of presenting multiple epitopes to an organism in need of said immunization. A plasmid for expression of multiple epitopes comprising a nucleic acid sequence encoding multiple epitopes, wherein each of said epitopes is capable of creating an immune response. A multivalent expression system as shown in Figure 8 and selected from the group consisting of two plasmids, two genes, IRES, and alternative splicing and a method of making a plasmid producing the appropriate nucleic acid sequence.

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DESCRIPTION

Expression Plasmids For Multiepitope Nucleic Acid-Based Vaccines

Statement of Related Applications

This application is related to U.S. Patent Application entitled "IL-12 Gene Expression and Delivery Systems and Uses", filed October 10, 1997, Serial No. not yet assigned, by Jeff Nordstrom, Bruce Freimark and Deepa Deshpande, Lyon & Lyon Docket No. 226/285 and U.S. Patent Application entitled "Gene Expression and Delivery Systems and Uses", filed October 10, 1997, Serial No. not yet assigned, by Jeff Nordstrom, Bruce Freimark and Deepa Deshpande, Lyon & Lyon Docket No. 226/284, both of which are incorporated herein by reference in their entirety, including any drawings.

Introduction

The invention relates generally to gene therapy, in particular, the invention relates in part to improved plasmids and methods for nucleic acid based vaccines.

Background of the Invention

The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

Plasmids are an essential element in genetic engineering and gene therapy. Plasmids are circular DNA molecules that can be introduced into bacterial cells by transformation which replicate autonomously in the cell. Plasmids allow for the amplification of cloned DNA. Some plasmids are present in 20 to 50 copies during cell growth,

and after the arrest of protein synthesis, as many as 1000 copies per cell of a plasmid can be generated. (Suzuki et al., *Genetic Analysis*, p. 404, (1989).)

Current non-viral approaches to human gene therapy require that a potential therapeutic gene be cloned into plasmids. Large quantities of a bacterial host harboring the plasmid may be fermented and the plasmid DNA may be purified for subsequent use. Current human clinical trials using plasmids utilize this approach. (Recombinant DNA Advisory Committee Data Management Report, December, 1994, *Human Gene Therapy* 6:535-548). Studies normally focus on the therapeutic gene and the elements that control its expression in the patient when designing and constructing gene therapy plasmids. Generally, therapeutic genes and regulatory elements are simply inserted into existing cloning vectors that are convenient and readily available.

Plasmid design and construction utilizes several key factors. First, plasmid replication origins determine plasmid copy number, which affects production yields. Plasmids that replicate to higher copy number can increase plasmid yield from a given volume of culture, but excessive copy number can be deleterious to the bacteria and lead to undesirable effects (Fitzwater, et al., *EMBO J.* 7:3289-3297 (1988); Uhlin, et al., *Mol. Gen. Genet.* 165:167-179 (1979)). Artificially constructed plasmids are sometimes unstably maintained, leading to accumulation of plasmid-free cells and reduced production yields.

To overcome this problem of plasmid-free cells, genes that code for antibiotic resistance phenotype are included on the plasmid and antibiotics are added to kill or inhibit plasmid-free cells. Most general purpose cloning vectors contain ampicillin resistance (β-lactamase, or *bla*) genes.

Use of ampicillin can be problematic because of the potential for residual antibiotic in the purified DNA, which can cause an allergic reaction in a treated patient. In addition, β-lactam antibiotics are clinically important for disease treatment. When plasmids containing antibiotic resistance genes are used, the potential exists for the transfer of antibiotic resistance genes to a potential pathogen.

Other studies have used the *neo* gene which is derived from the bacterial transposon *Tn*5. The *neo* gene encodes resistance to kanamycin and neomycin (Smith, *Vaccine* 12:1515-1519 (1994)). This gene has been used in a number of gene therapy studies, including several human clinical trials (Recombinant DNA Advisory Committee Data Management Report, December, 1994, *Human Gene Therapy* 6:535-548). Due to the mechanism by which resistance is imparted, residual antibiotic and transmission of the gene to potential pathogens may be less of a problem than for β-lactams.

In addition to elements that affect the behavior of the plasmid within the host bacteria, such as *E. coli*, plasmid vectors have also been shown to affect transfection and expression in eukaryotic cells. Certain plasmid sequences have been shown to reduce expression of eukaryotic genes in eukaryotic cells when carried *in cis* (Peterson, et al., *Mol. Cell. Biol.* 7:1563-1567 (1987); Yoder and Ganesan, *Mol. Cell. Biol.* 3:956-959 (1983); Lusky and Botchan, *Nature* 293:79-81 (1981); and Leite, et al., *Gene* 82:351-356 (1989)). Plasmid sequences also have been shown to fortuitously contain binding sites for transcriptional control proteins (Ghersa, et al., *Gene* 151:331-332 (1994); Tully and Cidlowski, *Biochem. Biophys. Res. Comm.* 144:1-10 (1987); and Kushner, et al., *Mol. Endocrinol.* 8:405-407 (1994)). This

can cause inappropriate levels of gene expression in treated patients.

Nucleic acid vaccines, or the use of plasmid encoding antigens, has become an area of intensive research and development in the last half decade. Comprehensive reviews on nucleic acid vaccines have recently been published [M.A. Liu, et al.(Eds.), 1995, *DNA Vaccines: A new era in vaccinology*, Vol. 772, Ann. NY. Acad. Sci., New York; Kumar, V., and Sercarz, E., 1996, *Nat. Med.* 2:857-859; Ulmer, J.B., et al., (Eds.) *Current Opinion in Immunology*; 8:531-536. Vol. 772, Ann. NY. Acad. Sci., New York]. Protective immunity in an animal model using plasmid encoding a viral protein was first observed in 1993 by Ulmer et al. [Ulmer, J.B., et al., 1993, *Science* 259:1745-1749]. Since then, several studies have demonstrated protective immunity for several disease targets and human clinical trials have been started.

Summary

The use of epitopes, small immunologically relevant protein sequences that are capable of inducing both cellular and humoral responses that result in a protective or therapeutic immune response against large and complex pathogens, is an attractive and amenable strategy provided by the present invention for incorporation into nucleic acid-based vaccines. If multiple epitopes are expressed in the context of a synthetic gene construct, immunity against many antigenic targets, multiple strain variants or multiple pathogens is possible. This disclosure describes the structures and characteristics of gene expression systems that are capable of expressing multiple epitopes.

Thus, in one aspect the invention provides a method of genetic immunization comprising the step of presenting multiple epitopes to an organism in need of said immunization.

In preferred embodiments, the multiple epitopes are presented with one or more augmenting cytokines and/or are presented with a delivery vehicle selected from the group consisting of cationic lipids, delivery peptides, and polymer based deliver systems.

In another aspect, the invention features a plasmid for expression of multiple epitopes comprising a nucleic acid sequence encoding multiple epitopes, wherein each of said epitopes is capable of creating an immune response.

In preferred embodiments, the plasmid includes a promoter, a 5' UTR sequence, and a 3' UTR sequence, a nucleic acid sequence encoding polyubiquitin, there are spacers between the nucleic acid regions encoding each of said epitopes, there are proteolytic cleavage sites between each of said epitopes, there are ER targeting signals between each of said epitopes, there are lysosomal and/or endosomal targeting sequences between each of said epitopes.

In other aspects, the invention provides a multivalent expression system as shown in Figure 8 and selected from the group consisting of two plasmids, two genes, IRES, and alternative splicing and a method of making a plasmid producing the appropriate nucleic acid sequence.

The summary of the invention described above is non-limiting and other features and advantages of the invention will be apparent from the following detailed description of the preferred embodiments, as well as from the claims.

Brief Description of The Drawings

Figure 1 shows a plasmid for multiple epitopes using a beads on a string approach.

Figure 2 shows a plasmid for multiple epitopes using beads on a string fused to polyubiquitin.

Figure 3 shows a plasmid for multiple epitopes using beads on a string with spacers between epitopes.

Figure 4 shows a plasmid for multiple epitopes using beads on a string with proteolytic cleavage sites between epitopes.

Figure 5 shows a plasmid for beads on a string epitopes with ER targeting sequences.

Figure 6 shows a plasmid for multiple epitopes with ER targeting sequences.

Figure 7 shows a plasmid for multiple epitopes with lysosomal/endosomal targeting sequences.

Figure 8 shows types of multivalent expression systems.

Figure 9 shows a DNA vaccine expression plasmid with two genes.

Figure 10 shows a design of a drug-controlled DNA vaccine expression plasmid.

Detailed Description of the Preferred Embodiments

Various exemplary plasmids and methods for multiepitope nucleic acid based vaccines are described below.

The following explanation of the invention is to aid in understanding various aspects of the invention. The following explanation does not limit the operation of the invention to any one theory.

I. Expression Plasmid for Epitopes Arranged as Beads-on-a-String

In expression plasmids of this type, the multiple epitopes are directly linked to each other. No spacer sequences between the epitopes are included. The epitope sequences themselves are sufficient for the formation of a functional "pseudo" protein that can be processed into individual peptide epitopes via proteosome cleavage. This concept, i.e. beads-on-a-string, is supported by data that shows full CTL responses to numerous epitopes when they are placed into novel locations within different proteins (Nomura M, Nakata Y, Inoue T *et al.*, *J. Immunol.* Methods, 193:41-9 (1996) and Weidt G, Deppert W, Buchhop S, Dralle H, Lehmanngrube F., *J. Virol.*, 69:2654-8 (1995)).

An and Whitton, *J. Virol.*, 71:2292-302 (1997) have described that a beads-on-a-string approach is feasible with a recombinant vaccinia virus vector. They appear to have demonstrated that a linear array of B-cell, CTL and Th epitopes was able to induce the corresponding immune response. Gilbert et al., *Nature Bio.*, 15:1280-84 (1997) has demonstrated that the beads-on-a-string approach is feasible with a recombinant Ty-VLP vector. They described that a linear array of 15 defined malaria epitopes induced protective CTL responses in mice, and that neither epitope order nor flanking sequences influenced the processing of the epitopes.

Multiple epitopes expressed from a recombinant vaccinia virus vector as a string of 10 contiguous minimal CTL epitopes, which were restricted by five MHC alleles and derived from five viruses, a parasite, and a tumor model, induced a primary CTL response *in vivo* in the appropriate mouse strain. This illustrates that multiple CTL epitopes

can be effectively delivered in a beads-on-a-string array (Thomson et al., *J. Immunol.*, 157:822-6 (1996)).

The present invention provides an exemplary expression system for beads-on-a-string as shown below (See Figure 1):

Promoter / 5' UTR / intron / AUG / $(Epitope)_n$ / stop codon / 3' UTR / poly(A) signal

Table I below provides a description of each of these genetic elements.

Table I. Description of genetic elements.

Element	Description
Promoter	CMV, tissue-specific (e.g. APC-specific), or synthetic promoter
5' UTR	Optimized to assure mRNA stability and translatability. Current optimal sequences are UT11 (from human loricrin gene) or UT12 (from CMV).
Intron	Synthetic intron that has optimized 5' ss, 3' ss and branch point sequences. Current optimal sequence is IVS 8.
Initiation codon	AUG is placed in the context of the Kozak sequence to ensure optimal initiation of translation.
$(Epitope)_n$	String of epitopes, each having a length of 9-10 amino acid residues in length for class I presentation, or >10 amino acid residues for class II presentation. It appears that at least 15 epitopes may be strung together. One of the main considerations will be to avoid the placement of glycine or proline adjacent to the desired epitope termini.

Stop codon	For termination of translation. To ensure efficient termination, it is desirable to string two stop codons in tandem.
3' UTR/poly(A) signal	To ensure efficient processing of the mRNA an efficient poly(A) signal, such as from human growth hormone, is required.

II. Expression Plasmid for Multiple Epitopes as Beads-on-a-String Linked to a Polyubiquitin Chain

Degradation of many eukaryotic proteins requires their prior ligation to polyubiquitin (Ub) chains, which target the substrates to the 26S proteasome, an abundant cellular protease. Thus, it is advantageous to encode a Ub chain that is fused to the N-terminus of the multiple epitopes that are arranged as beads-on-a-string. Expression plasmids with Ub fused to the antigen have been used to achieve class I presentation (Gueguen and Long, *Proc. Natl. Acad. Sci. USA* 93:14692-97 (1996)).

Illustrated below is an expression system for beads-on-a-string fused to polyubiquitin (Ub) (See Figure 2):

Promoter / 5' UTR / intron / AUG / Ub / $(Epitopes\text{-}Spacer)_n$ / stop codon / 3' UTR / poly(A) signal

III. Expression Plasmid for Multiple Epitopes as Beads-on-a-String with Spacers Between Epitopes

An expression system for beads-on-a-string with spacers is shown below (See Figure 3):

Promoter / 5' UTR / intron / AUG / $(Epitopes\text{-}Spacer)_n$ / stop codon / 3' UTR / poly(A) signal

Some investigators have shown that flanking sequence can profoundly influence the generation of epitopes (Yellenshaw et al., *J. Immunol.*, 158:1727-33 (1997); Shastri et al., *J. Immunol.*, 155:4339-46 (1995); Del-Val et al., *Cell*, 66:1145-93 (1991); Eggers et al., *J. Exp. Med.* 182:1865-70 (1995); Niedermann et al. *Immunity*, 2:289-95 (1995); Lippolis et al. *J. Virol.* 69:3134-46 (1995)). In particular, glycine or proline residues adjacent to the minimal epitope should to be avoided, since peptide bonds to these residues are known to be resistant to protease activity (Niedermann et al., 1995).

Spacers may facilitate the formation of epitopes that induce immunity. Ideally, the spacer sequence should be one that does not conform at all to the rules for class I or II epitope. However, it may be desirable to include a hydrophobic, basic or acidic residue at the C-terminus of the spacer to facilitate cleavage between the spacer and the adjacent epitope. The length of the spacer that would be optimal is not known and would have to be determined empirically.

IV. Expression Plasmid for Multiple Epitopes as Beads-on-a-String with Proteolytic Cleavage Sites Between Epitopes

An expression system for beads-on-a-string with proteolytic cleavage sites is diagramed in summary form below (See Figure 4):

Promoter / 5' UTR / intron / AUG / (Epitopes-Cleavage Site)$_n$ / stop codon / 3' UTR / poly(A) signal

Many viruses (e.g. retroviruses, flaviviruses) generate mRNAs that encode polyproteins that must undergo proteolytic cleavage to form the mature protein products. Cleavage, which occurs at specific sites, is catalyzed by host

proteinases or by virally encoded proteinases. For example, the polyprotein from hepatitis C virus is structured as follows: H2N-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Host cell signal peptidases cleave the junctions in the region between C and NS2. The viral proteinase (NS2-3 proteinase) cleaves the junction between NS2 and NS3. Another viral proteinase (NS3 proteinase) cleaves the junctions between NS3 and NS5B.

One approach is to insert host cell cleavage sites between the epitope sequences. This may be achieved by insertion of the sequences that are located at the junctions between the C-E1-E2-p7-NS2 proteins of the hepatitis polyprotein. Other possibilities are to utilize the recognition site for specific cellular proteases.

A second approach is to insert cleavage sites for the viral proteinase between the epitope sequences. Thus, the site for the sequence recognized by the NS3 proteinase, Asp/Glu-X-X-X-Cys/Thr↓Ser/Ala (Koch and Bartenschlager, *Virology*, 237:78-88 (1997)), may be inserted. However, for this approach to work, the NS3 proteinase must be also encoded by the expression plasmid. Thus, two transcription units are required, one for the multiepitopes, one for the viral proteinase. See Section V. Multivalent expression plasmids for nucleic acid-based vaccines.

V. Expression Plasmids for Multiple Epitopes With Targeting

The classical pathway for antigen presentation in the context of class I involves the partial degradation of antigenic proteins into peptides by the proteasome. The peptides are then transported into the endoplasmic reticulum by peptide transporters (TAP-1 and TAP-2). It is within the

lumen of the ER or cis-Golgi that the peptides are loaded into the binding pocket of the MHC class I molecules. Ciernik et al., *J. Immunol.* 2369-75 (1996) have demonstrated that the immunogenicity of an epitope may be enhanced if the epitope sequence is fused in frame with the adenovirus E3 leader sequence and expressed from a plasmid delivered by particle bombardment. An epitope fused to the E3 leader yielded greater protection from tumor challenge than an epitope without the leader.

ER targeting signals have also been used by Overwijk et al., Identification of a Kb-restricted Ctl Epitope of Beta-galactosidase: Potential Use in Development of Immunization Protocols for "Self" Antigens, Methods **12:** 117-23 (1997). The signal sequence allows the epitope to be targeted to the ER by the standard protein translocation process. The N-terminal leader sequence targets the peptide to the ER by first binding, as a nascent sequence, to the 54 kDa subunit of the SRP particle. The leader sequence subsequently binds to the b-subunit of the membrane-bound transporter protein, Sec61p. Following entry into the lumen of the ER through a putative channel, a peptidase cleaves the peptide to remove the leader sequence. This mechanism is independent of the TAP transporter system. This alternative mechanism may be advantageous if epitope formation by proteasome cleavage, or epitope transport by the TAP system, are limiting steps in antigen presentation.

For ER targeting, a leader sequence preferably needs to be attached to the N-terminus of each epitope. Adding a leader sequence to the multiple epitopes that are arranged as beads-on-a string concept is unlikely to work, since the leader will be attached only to the first epitope sequence.

Placing an individual targeting sequence on each of the epitopes that is arranged in a bead-on-a-string assembly is a possibility. However, this strategy will depend on accurate proteolytic cleavage at the N-terminus of the leader sequence and at the C-terminus of the adjoined epitope sequence. A gene expression system that utilizes alternative splicing will yield individual epitopes with their own leader sequences. The peptide epitopes produced by this strategy will not depend on random degradation of a protein precursor.

The only processing that is required is N-terminal processing that is associated with protein translocation. The C-terminal ends of the epitopes are defined by the stop codons that are designed into the system. The preprotein products may be incompletely synthesized until protein translocation through the pore into the ER has occurred. Alternatively, the prepeptides may be synthesized in their entirety prior to ER translocation. This may expose the prepeptide to the proteasome and transport of proteins that transport epitopes to the ER by the standard pathway.

A. Expression Plasmid for Multiple Epitopes with ER Targeting Sequences

An expression system for beads-on-a-string epitopes with targeting sequence is shown below (See Figure 5):

Promoter / 5' UTR / AUG / ER signal sequence / epitope-1 / stop codon / ER signal sequence / epitope-2 / stop codon / ER signal sequence / epitope-3 / stop codon / 3' UTR/poly(A) signal

An alternative splicing system for multiple epitopes with targeting sequence is shown below (See Figure 6):

Promoter / 5' UTR / AUG / ER signal sequence / 5'ss / internal intron sequence / 3'ss-1 / epitope-1 / stop codon-1

/ 3'ss-2 / epitope-2 / stop codon-2 / 3'ss-3 / epitope-3 / stop codon-3 / 3' UTR/poly(A) signal

Table II below describes the genetic elements used in the alternative splicing strategy.

Table II. Description of genetic elements for the alternative splicing strategy.

Element	Description
ER Signal sequence 5' ss	The N-terminal leader sequence from adenovirus E3 or preprolactin Strong 5' splice site, one that exactly matches the consensus sequence. Such a sequence is found in the synthetic intron, IVS8.
Internal intron sequence	This sequence is derived from the synthetic intron, IVS8. It extends from the 3' end of the 5' splice site to the 5' end of the polypyrimidine tract of the 3'splice site.
Alternative 3' splice sites	3' ss-1, 3' ss-2, 3' ss-3: These splice sites will be designed to be used equally. Thus, their relative strengths need to be mathed. This will be accomplished by introducing purines within the polypyrimidine regions of the splice site sequences.

In the alternative RNA splicing system, the strengths of the 3' splice sites must be balanced to splicing from the 5'ss to each of the 3' splice sites (3'ss-1, to 3'ss-2, to 3'ss-3, etc.). Balanced splicing will be achieved by controlling the purine content of the pyrimidine-rich

sequences of the 3' splice sites. In general, the greater the purine content, the weaker the splice site. There are model systems to follow. For example, the major late transcript of adenovirus is alternately spliced into 5 families of transcripts that are produced in roughly equivalent amounts. Thus, one way to design an appropriate alternatively spliced system for epitopes is to model the 3' splice sites of adenoviral late transcripts.

Another key feature is that, after splicing, the leader sequence must be fused in frame with the peptide sequence of each epitope. Also, by altering the strengths of the 3' splice sites, the relative amounts of the epitopes may be varied. This may important if certain epitopes are more dominant than others.

Table III below shows an example of a balanced set at 3' splice sites derived from the adenoviral late transcript.

Table III. Example of a balanced set of 3' splice sites (derived from the adenoviral late transcript)

5′ ss	Source	Alternative 3′ ss[1/]	Source[2/]	
CAG↓GTAAGT	IVS8	TTTGCTTTTCCCCAG↓G	Ad2	(11039)
	Consensus 5′ ss)			
		TTGTATTCCCCTTAG↓T	Ad2	(14149)
		GTTGTATGTATCCAG↓C	Ad2	(16515)
		GTAACTATTTTGTAG↓A	Ad2	(17999)
		CCATGTCGCCGCCAG↓A	Ad2	(18801)
		ATGTTTTGTTTGAAG↓T	Ad2	(21649)
		TTCCTTCTCCTATAG↓G	Ad2	(24094)

1/ The consensus sequence for a 3' ss is YYYYYYYYYYYNYAG↓G. Y = C or T, and ↓ = intron/exon junction.

2/ Adenovirus 2 (Ad2) sequences are from the Genbank entry, ADRCG. Numbers in parentheses indicate the nucleotide position of each 3' splice site. Note the locations of the purines (A or G) that interrupt the polypyrimidine (C or T) region.

B. Expression Plasmid for Multiple Epitopes with Endosomal/Lysosomal Targeting Sequences

An expression system structure is shown below (See Figure 7):

Promoter / 5' UTR / AUG / ER signal sequence / 5'ss / internal intron sequence / 3'ss-1 / epitope-1 / LAMP-1 transmembrane-cytoplasmic tail / stop codon-1 / 3'ss-2 / epitope-2 / LAMP-1 transmbrane-cytoplasmic tail / stop codon-2 / 3' ss-3 / epitope-3 / LAMP-1 transmbrane-cytoplasmic tail / stop codon-3 / 3' UTR/poly(A) signal

To target class II antigen presentation, it may be desirable to directly target the peptide epitope to the endosomes or lysosomes. One strategy employs the transmembrane and cytoplasmic tail sequences from a one of the lysosomal-associated membrane glycoproteins, such as LAMP-1. Wu et al., (1995) have used such a sequence, in combination with an N-terminal ER targeting sequence, to target an antigen to the endosomal and lysosomal compartments for class II antigen presentation. Thus, each epitope is preceded by an N-terminal leader sequence (e.g. adenovirus E3) and followed by the C-terminal endosomal/lysosomal targeting sequence (e.g. the transmembrane and cytoplasmic tail region of LAMP-1). Another sequence that may be employed for endosomal targeting is the cytoplasmic tail of membrane immunoglobulin (Weiser et al., *Science* 276:407-9 (1997); Achatz et al., *Science* 276:409-11 (1997)).

Since the transmembrane/cytoplasmic tail can be added to some, but not necessarily all epitopes, it would be possible to target some epitopes to the ER for class I presentation and others to the endosomes/lysosomes for class II presentation.

VI. Multivalent Expression Plasmids for Nucleic Acid-Based Vaccines

For effective nucleic acid-based vaccines, it may be important to have the capability of expressing multiple gene products. For example, expression of multiple intact antigens or multiepitope gene product may enhance the potency of the these vaccines. Co-expression of costimulatory proteins, such as IL-2, IL-6, IL-12, GM-CSF, B7.1 or B7.2, have been demonstrated to enhance the immune response to an encoded antigen (Geissler et al., *J. Immunol.* 158:1231-1237 (1997), Irvine et al., (1996); Kim et al., *Vaccine* 15:879-83 (1997); Okada et al., *J. Immunol.* 159:3638-47(1997); Barry and Johnston, Scand. J. Immunol., 45:605-12(1997)). Co-expression of proteins that facilitate peptide epitope formation, such as proteolytic enzymes (e.g. the NS3 protease from hepatitis C (Koch and Bartenshlager, 1997)) or chaperone proteins (e.g. heat shock protein Hsp65 (Wells et al., *Scand. J. Immunol.*, 45:605-12 1997)), may also enhance the response.

The various types of multivalent expression plasmids are described in Figure 8. They include (1) multiple complete genes, or transcription units, on a single plasmid, (2) generation of polycistronic mRNAs using a internal ribosome entry site (IRES) sequence, and (3) generation of multiple mRNAs by alternative RNA splicing. The design of a nucleic acid-based vaccine expression plasmid that has two genes is shown in Figure 9.

The details of these systems are described in the related patent applications incorporated herein by reference on page 1.

Examples

The present invention will be more fully described in conjunction with the following specific examples which are not to be construed in any way as limiting the scope of the invention.

Geneswitch Example

The GeneSwitch is a chimeric protein that consists of human progesterone receptor with a modified ligand binding domain, a DNA binding domain from yeast GAL4, and an activator domain from Herpes Simplex VP16. When a synthetic steroid, mifepristone, is administered, the GeneSwitch protein becomes activated (binds the synthetic steroid, presumably dimerizes, and translocates to the nucleus). The activated GeneSwitch then binds to a target sequence (multiple GAL4 binding sites linked to a minimal promoter) and thereby stimulates the transcription of the desired transgene (Wang et al., *Proc. Natl. Acad. Sci. USA* 91:8180-84 (1994); Wang et al., *Nature Biotechnology* 15:239-243 (1997a); Wang et al., *Gene Therapy* **4**:432-41 (1997b)).

The GeneSwitch may be used to regulate the expression of a plasmid for nucleic acid-based vaccines. It is possible that the timing of expression may influence the immune response. Thus, with a GeneSwitch regulated system, the genes that encode the multiepitopes may be turned on at a defined time after DNA delivery by the administration of the ligand (mifepristone) to the animal. If the expression plasmid persists *in vivo* for a long enough time, the GeneSwitch system also can be used to provide pulsatile expression of the multiepitope gene products. An example of the design of a system that is regulated by the GeneSwitch is shown in Figure 10.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any

equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example, if X is described as selected from the group consisting of bromine, chlorine, and iodine, claims for X being bromine and claims for X being bromine and chlorine are fully described.

Other embodiments are within the following claims.

Claims

1. A method of genetic immunization comprising the step of presenting multiple epitopes to an organism in need of said immunization.

2. The method of claim 1 wherein said multiple epitopes are presented with one or more augmenting cytokines.

3. The method of claim 1 wherein said multiple epitopes are presented with a delivery vehicle selected from the group consisting of cationic lipids, delivery peptides, and polymer based deliver systems.

4. A plasmid for expression of multiple epitopes comprising a nucleic acid sequence encoding multiple epitopes, wherein each of said epitopes is capable of creating an immune response.

5. The plasmid of claim 4 further comprising a promoter, a 5' UTR sequence, and a 3' UTR sequence.

6. The plasmid of claim 5 further comprising a nucleic acid sequence enclosing polyubiquitin.

7. The plasmid of claim 5 wherein there are spacers between the nucleic acid regions encoding each of said epitopes.

8. The plasmid of claim 5 further comprising proteolytic cleavage sites between each of said epitopes.

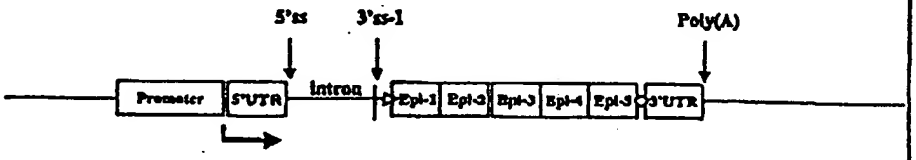
9. The plasmid of claim 5 further comprising ER targeting signals between each of said epitopes.

10. The plasmid of claim 5 further comprising lysosomal and/or endosomal targeting sequences between each of said epitopes.

11. A multivalent expression system as shown in Figure 8 and selected from the group consisting of two plasmids, two genes, IRES, and alternative splicing.

12. A method of making a plasmid of anyone of claims 4-10 comprising the step of producing the appropriate nucleic acid sequence.

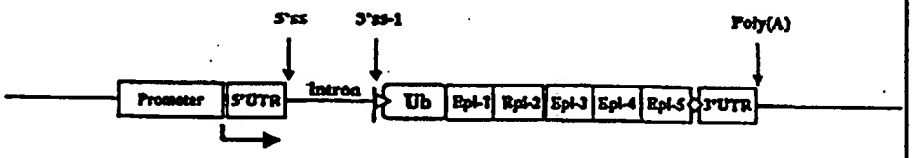
Fig. 1. Plasmid for Multiple Epitopes Using a Beads-on-a-String Approach



▷ Start codon

◇ Stop codon

Fig. 2. Plasmid for Multiple Epitopes Using Beads-on-a-String Fused to Polyubiquitin



▷ Start codon

◇ Stop codon

Ub Polyubiquitin

Fig. 3. Plasmid for Multiple Epitopes Using Beads-on-a-String with Spacers Between Epitopes

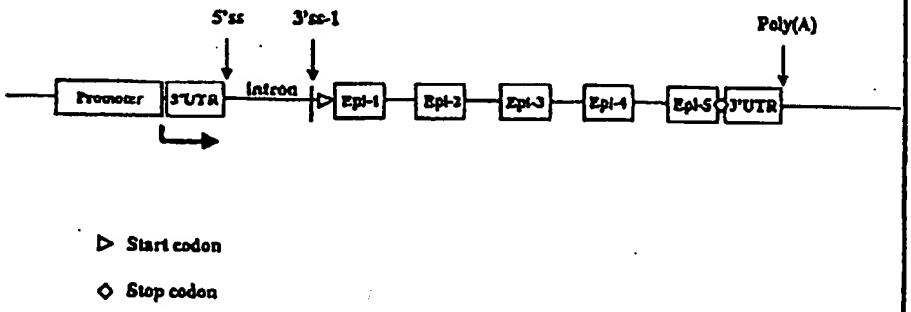


Fig. 4. Plasmid for Multiple Epitopes Using Beads-on-a-String with Proteolytic Cleavage Sites Between Epitopes

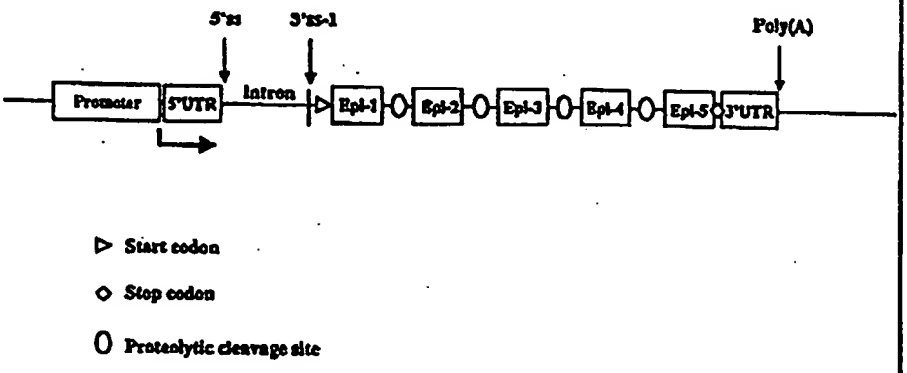


Fig. 5. Plasmid for Beads-on-a-String Epitopes with ER Targeting Sequences

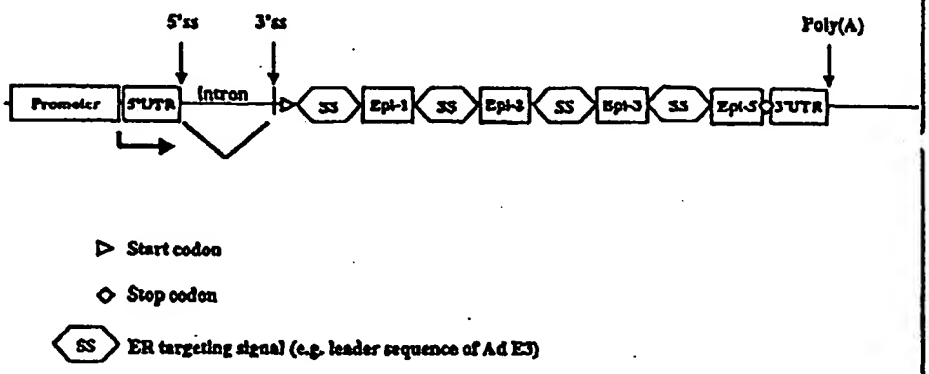


Fig. 6. Plasmid for Multiple Epitopes with ER Targeting Sequences

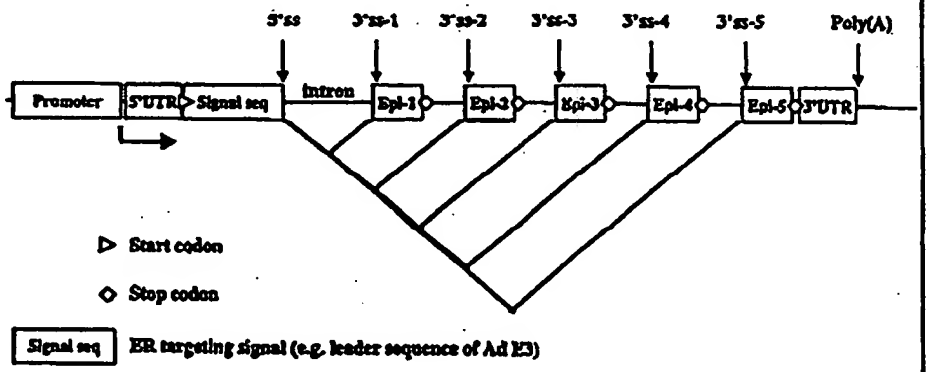


Fig. 7. Plasmid for Multiple Epitopes with Lysosomal/Endosomal Targeting Sequences

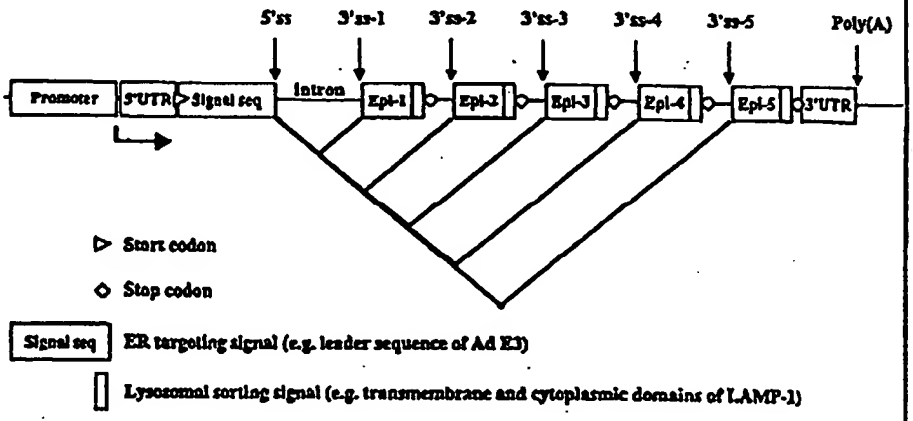


Fig. 8. Types of Multivalent Expression Systems

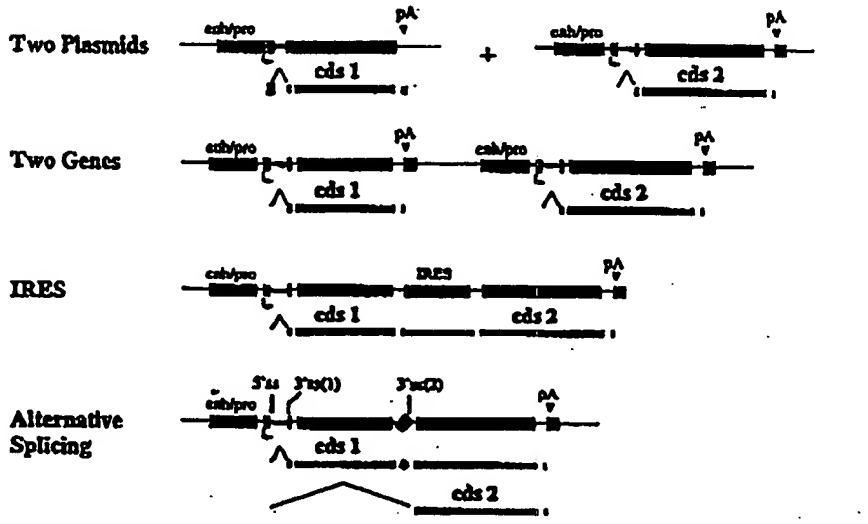


Fig. 9. DNA Vaccine Expression Plasmid with Two Genes

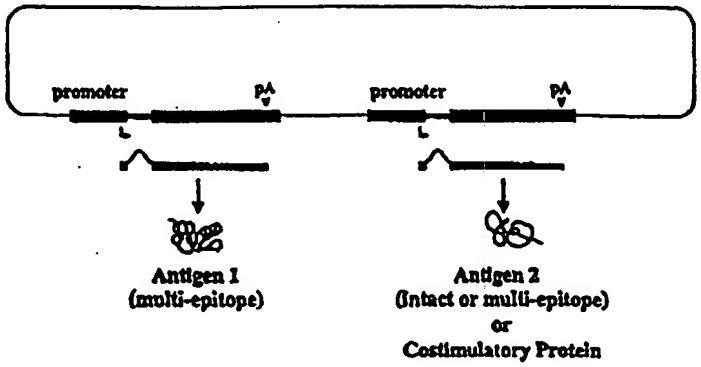
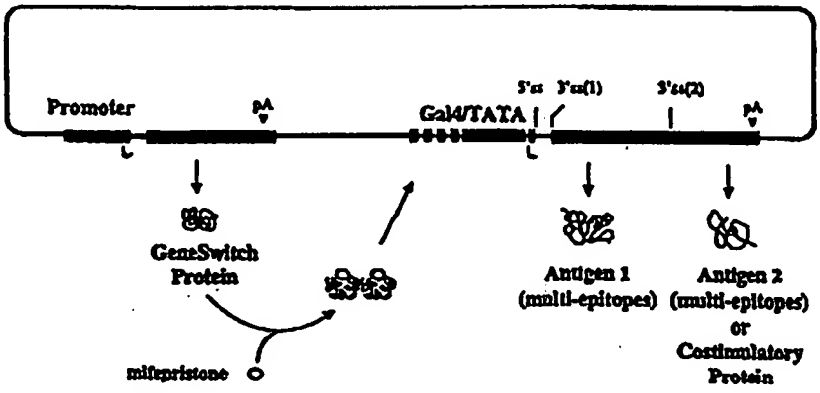


Fig. 10. Design of a Drug-Controlled DNA Vaccine Expression Plasmid



INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 98/23745

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 35021 A (US HEALTH ;ZAREMBA SAM (US); SCHLOM JEFFREY (US); TSANG KWONG YOK) 25 September 1997	1-5,7-9, 12
A	see the whole document	11
X	WO 96 03144 A (QUEENSLAND INST MED RES ;COMMENWEALTH SCIENT AND IND RES (AU)) 8 February 1996	1-4,12
A	see the whole document	5-11

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 April 1999	19/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/23745

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 24870 A (BIOTRANSPLANT INC ;GEN HOSPITAL CORP (US); SEED BRIAN (US); BANERJ) 10 November 1994	1-4,11, 12
A	see abstract see page 2 - page 4 see page 11 - page 14 see examples 1-4 see figure 1 see claims	5-10
X	DE 195 14 310 A (KLINIKUM DER ALBERT LUDWIGS UN) 24 October 1996 see abstract see column 1, line 68 - column 2, line 62	11
X	WO 91 02805 A (VIAGENE INC) 7 March 1991 see abstract see page 6, line 19 - line 37 see page 7, line 15 - line 21 see page 13, line 35 - page 14, line 14 see examples 2B,,2E,,2F see page 75, line 11 - line 26 see claims 1,6	1-4,11, 12

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 23745

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-10 and 12 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter. nal Application No
PCT/US 98/23745

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 5851529 A	22-12-1998
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